Nutrition and Disease

Walnuts Reduce Aortic ET-1 mRNA Levels in Hamsters Fed a High-Fat, Atherogenic Diet[1,2]

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ABSTRACT   Walnut consumption is associated with reduced coronary vascular disease (CVD) risk; however, the mechanisms responsible remain incompletely understood. Recent clinical studies suggested that these mechanisms involve nonplasma lipid–related effects on endothelial function. Male Golden Syrian hamsters (12 groups, $n = 10$–15) were fed for 26 wk atherosclerotic, high-fat, hyperlipidemic diets with increasing concentrations of whole walnuts (61–150 g/kg diet), or α-tocopherol (α-T, 8.1–81 mg/kg diet) and single diets with either walnut oil (32 g/kg diet) or pure γ-tocopherol (γ-T; 81 mg/kg diet) added. Aortic endothelin 1 (ET-1), an important endothelial regulator, was assayed as mRNA. Aortic cholesterol ester (CE) concentration along with other vascular stress markers (Cu/Zn and Mn superoxide dismutase, biliverdin reductase) and plasma lipid concentrations were determined. Hyperlipidemia (plasma LDL cholesterol ~6 times normal) occurred in all groups. Aortic CE concentration, a measure of athero-sclerotic plaque, was highest in the lowest α-T only group and declined significantly with increasing α-T. The aortic CE of all walnut groups was decreased significantly relative to the lowest α-T only group but showed no dose response. The diets did not produce changes in the other vascular stress markers, whereas aortic ET-1 mRNA levels declined dramatically with increasing dietary walnuts (to a 75% reduction in the highest walnut content group compared with the lowest α-T group) but were unaltered in the α-T groups or γ-T group. The study results are consistent with those of human walnut feeding studies and suggest that the mechanisms underlying those results are mediated in part by ET-1–dependent mechanisms. The contrasting results between the α-tocopherol or γ-tocopherol diets and the walnut diets also make it unlikely that the nonplasma lipid–related CVD effects of walnuts are due to their α-tocopherol or γ-tocopherol content. Finally, the results indicate that the walnut fat compartment is a likely location for the components responsible for the reduced aortic CE concentration.   J. Nutr. 136: 428–432, 2006.

KEY WORDS: • walnuts • endothelin • hamsters • tocopherol • atherosclerosis

Walnuts are rich in (n-6) and (n-3) PUFA and phytochemicals; their consumption is linked to reduced coronary vascular disease (CVD)[4] risk by a U.S. FDA–approved limited health claim (1). However the factor(s) responsible for CVD risk reduction, as with many dietary interventions, remain incompletely understood. Recent studies by Ros et al. (2). and Zhao et al. (3) using walnuts as a fat source suggested that the CVD risk reduction upon walnut consumption may be related to nonplasma lipid–related effects on endothelial function.

The current study assessed the effect of whole walnuts on atherosclerosis in hypercholesterolemic Golden Syrian hamsters in vivo; these hamsters have CVD similarities to humans, i.e., their response to an atherogenic diet and the development of atherosclerosis-associated lesions. We used this model previously to demonstrate that in the presence of low dietary tocopherol, catechin reduces atherosclerotic plaque deposition (4). CVD is linked to oxidative stress and inflammatory mediators (5), and endothelin 1 (ET-1) has been closely associated with plaque development, smooth muscle proliferation, and remodeling (6). Human atherosclerotic plaque smooth muscle cells and macrophages have enhanced ET-1 expression (7–9), and ET-1 expression is stimulated and controlled at the transcription level by oxidative stress (10,11). Importantly, ET-1 receptor (ETA receptor) blockade inhibits lesion development in experimental atherosclerosis (12–14), and ET-1 expression can be altered by tocopherol (15).

The study employed a series of hypercholesterolemic mixed-lipid semipurified diets with increasing levels of either walnuts or tocopherol. The hamsters were fed the diets for 26 wk and the responses of atherosclerosis related markers, ET-1, stress markers such as Cu/Zn and Mn superoxide dismutase (SOD), and aortic cholesterol ester (CE) concentration to differing levels of walnuts, α-tocopherol (α-T), and γ-tocopherol (γ-T) were assessed.

MATERIALS AND METHODS

Animals. Male Golden Syrian hamsters (21 d old; Sasco strain, Charles River Laboratories) were fed a laboratory rodent diet (no. 5001, Ralston Purina) for 2 wk. Hamsters were then weighed and assigned by weight to 1 of 12 experimental dietary groups ($n = 10$–15 hamsters/group). The hamsters were housed individually in wire-bottomed cages in an environmentally controlled room (20–22°C, 60% relative humidity, 12-h alternating light:dark cycle). The diets were fed for 26 wk with diet consumption measured 2 times/wk and animal weight every week. The protocol for use of hamsters was approved by the Animal Care and Use Committee, USDA Western Regional Research Center, Albany, CA.

Diets. The diets consisted of 100 g of fat (80% butterfat, 20% fish oil with 2% cholesterol); 100 g vitamin E–stripped soybean oil solely, or a series of weight equivalent mixtures of vitamin E–stripped soy oil; and walnuts or walnut oil that totaled 100 g of fat with 800 g of a powdered, fat- and vitamin E–free AIN-76A purified diet base (16), adjusted to accommodate the protein and carbohydrate content of the added walnuts. Diet components had minimal vitamin E levels (tocopherol-stripped oils); the diets were then formulated with increasing levels of α-T by the addition of pure α-T. Another 2 diets had increasing levels of γ-tocopherol (γ-T) using 2 different sources of γ-T (one as pure γ-T, the other as γ-T in walnut oil). Differences in fatty acid composition were avoided by using vitamin E–stripped soybean oil as a fat source to accommodate as much as possible the changes introduced by adding increasing amounts of walnuts. Diets were virtually identical in fatty acid composition as determined using both food composition data and GC analysis. The tocopherol levels of

the various diets were calculated from food composition data (**Table 1**) and HPLC-based tocopherol analysis (17); they were stable for 1 wk of exposure under the same conditions as when presented to the hamsters (data not shown).

Tissue and plasma samples. Hamsters were killed under anesthesia by diaphragm puncture after midline incision. Blood was obtained by immediate cardiac puncture using EDTA-treated syringes. Plasma was obtained after centrifugation (1500 × g, 30 min at 4°C) and aliquots were stored at −80°C before analysis. Liver was removed, weighed, and flash frozen at −196°C. The heart and great vessels were then removed from the chest cavity and the aorta of each hamster was harvested by dissection. The aorta was removed, cleaned, and slit lengthwise along the outside of the arch to expose the interior. The aorta was then divided into samples for immunoblotting, mRNA levels, and cholesterol determinations with care taken to ensure that samples for each particular assay were similar in location and size. Whole vessels were used as described by Schiffrin et al. (18) because Matawari et al. (19) and others (20,21) reported that ET-1 was produced in the whole aorta as well as in other associated tissues and all may exert control over arterial function via ET-1. Samples designated for mRNA isolation were stored in RNALater (Ambion) for later processing, which occurred ~2 wk after the first hamster was killed. The other samples were flash frozen at −196°C and stored at −80°C until used.

Plasma lipoprotein and cholesterol determinations. Plasma lipoproteins were separated, and cholesterol was measured using HPLC as previously described (22).

mRNA isolation. Aortic samples for mRNA were pooled by diet group, and mRNA was then isolated from each group of aortic samples using TriZol as described by the manufacturer (Invitrogen).

RT-PCR real-time quantitative PCR. The aortic ET-1 mRNA levels were measured by real-time quantitative PCR using SYBR Green quantitative PCR (Perkin Elmer Applied Biosystems) and ABI Prism 7700 Sequence Detector (Perkin Elmer). Primers were synthesized according to published cDNA sequences for ET-1 (23), and gylceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as internal controls. No-template (water) reaction mixture was used as a negative control. Quantitative analysis of PCR products was done by measuring in real time the increase in fluorescence intensity caused by SYBER Green dye binding to product. PCR data

TABLE 1

Composition of the semipurified diets[1]

Base diet component	Carbohydrate	Protein	Fat	Fiber	Vitamin/Mineral (mix − vitamin E)	
g/kg diet	498	200	200[2]	50	51	

		α-T		γ-T	Walnuts
Diet group	*n*	*mg/kg diet*		*mg/kg diet*	*g/kg diet*
AT1	15	8.1		—	—
AT2	15	16.2		—	—
AT3	15	32.4		—	—
AT4	15	81		—	—
WW1	15	3.35		17.70	61
WW2	10	3.57		21.76	75
WW3	10	3.74		24.95	86
WW4	10	3.95		29.01	100
WW5	10	4.34		36.27	125
WW6	15	4.73		43.52	150
GT	15	2.4		81	—
WO	15	3.35		11.33	32[3]

[1] Mean values ± SD for fatty acid composition of the AT1, WW1, and WW6 diets were as follows: 14:0, 5.5 ± 0.4%; 16:0, 19.8 ± 0.7%; 16:1, 1.9 ± 0.14%; 18:0, 7.9 ± 0.3%; 18:1, 19.8 ± 1.8%; 18:2 30.9 ± 3.0%; 18:3 6.4 ± 0.5%; 20:5 1.2 ± 0.12%; 22:0 1.1 ± 0.8%.
[2] Contained vitamin E–stripped soy oil, butterfat, fish oil blended 5:4:1, 2 g of cholesterol.
[3] Added as walnut oil; equivalent to the fat content of 61 g of whole walnuts.

were analyzed by the Sequence Detector v1.6.3 program (Perkin Elmer), and ET-1 mRNA expression was normalized using GAPDH mRNA expression levels. The mRNA level analysis was done in triplicate and repeated.

Western blot. For Western blot analysis, proteins were extracted as described by Valacchi et al. (24) from each of the pooled diet group samples [120 μg protein, determined using Bradford protein assay (Biorad)], separated on 4–20% gradient SDS-PAGE gels, and electrotransferred onto nitrocellulose membranes. After being blocked in 3% nonfat milk in PBS, the membranes were probed with 1 mg/L of specific polyclonal antibodies against Mn SOD, Cu/Zn SOD, and biliverdin reductase (Stressgen Biotechnologies). Goat anti-rabbit horseradish peroxidase-conjugated antibody (Sigma) was used as a secondary antibody, visualized by enhanced chemiluminescence (ECL Detection Kit, Amersham), and captured on X-ray film. Band densities were quantitated using NIH Image shareware.

Aortic lesions: aortic free and total cholesterol determination. As a marker of aortic atherosclerosis induced by the hyperlipidemia, CE concentrations in the aortic arch were determined (25). The tissue samples were cleaned and then extracted using chloroform:methanol (2.5:1) at room temperature for 48 h. The tissues were then removed; Triton-100 (1% in CHCl$_3$) was added after the volume was reduced under N$_2$, mixed, and evaporated completely at 37°C under N$_2$. The lipid extract was then solubilized in distilled water by mixing on a vortex and placing in a shaking water bath at 37°C for 20 min. Aortic total and free cholesterol concentrations were then determined enzymatically (Free Cholesterol C and Cholesterol E assay kits; Wako Bioproducts) and aortic CE was calculated as the difference between the total and free cholesterol. Aortic protein was determined on the basis of nitrogen content using combustion analysis (vario MACRO, Elementar Americas) and used to adjust for differing tissue sample sizes.

Statistical methods. Results for data derived from individual animals were analyzed using ANOVA; where appropriate, post hoc comparisons were made using the Bonferroni post hoc comparison method. *P*-values < 0.05 were considered significant. The analyses of the results for the ET-1 mRNA were done using a combination of randomization (26–28) and rank tests given that a single pool of ET-1 mRNA per treatment group was used for analysis, which limits estimation of underlying uncertainties due to variation among hamsters. The randomization tests were done using the Resample Macro addon for Excel spreadsheets (Resampling Stats) and *P*-values < 0.05 were considered significant.

RESULTS

Food intake and growth. The different dietary groups did not differ in food intake or body weight over the course of or at the end of the study (data not shown).

Lipoprotein cholesterol concentrations. All hamsters were grossly hyperlipidemic with elevated plasma LDL cholesterol ranging from 4.93 ± 1.02 to 8.67 ± 1.86 mmol/L (normal hamster LDL cholesterol is ~1.15 mmol/L) (data not shown). The groups did not differ and there was no relation by regression analysis between either plasma LDL levels and diets fed or between plasma LDL levels and any of the markers assessed.

Aortic Mn SOD, Cu/Zn SOD and biliverdin reductase protein levels. The aortic levels of Mn SOD, Cu/Zn SOD and biliverdin reductase proteins were assayed by Western blot, which demonstrated that Mn SOD, Cu/Zn SOD and biliverdin reductase levels, while demonstrable, exhibited no diet-related differences (data not shown).

Aortic esterified cholesterol. Aortic CE concentration as a marker for aortic atherosclerosis was affected by the diets (*P* < 0 0.0001). Hamsters fed diets with increasing α-T added had aortic CE concentrations that declined as α-T increased; the lowest α-T diet group (AT1; CE level 2.65 ± 0.44 nmol/g protein) was significantly higher than any of the CE means from the remaining α-T diet groups (AT2–4) (**Fig. 1**). In addition,
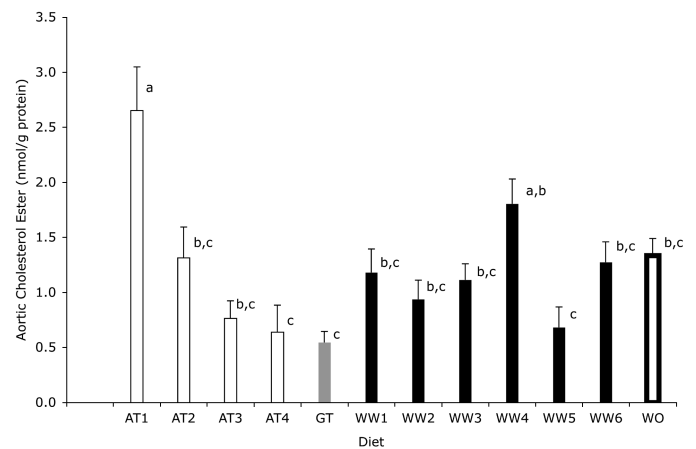

FIGURE 1 Aortic esterified cholesterol concentrations in hamsters fed high-fat diets containing various combinations of walnuts and tocopherols. Values are means ± SEM, *n* = 10 or 15 (means of duplicate determinations from individual hamsters). Means without a common letter differ, *P* < 0.05. Esterified cholesterol was calculated as the difference between total and free cholesterol and was expressed relative to aortic protein content.

aortic CE concentration in the pure γ-T diet group GT (0.54 ± 0.11) was significantly lower than that of the 2 lowest α-T added diet groups (AT1 and AT2). Diet groups with added walnut oil (WO) or whole walnuts (WW1–6) all had lower aortic CE concentration than that of the lowest α-tocopherol diet group, AT1 (*P* < 0.01). However, increasing the levels of walnuts did not reveal a dose response because aortic CE concentration did not differ with increasing walnut content.

ET-1 mRNA. The changes in aortic ET-1 mRNA levels as relative amounts (normalized to GAPDH) in hamsters fed diets with increasing amounts of either pure α-T or γ-T (**Fig. 2**) were not significant. The highest level of ET-1 mRNA occurred in the aortic tissue of hamsters in the AT1 diet group. In contrast,
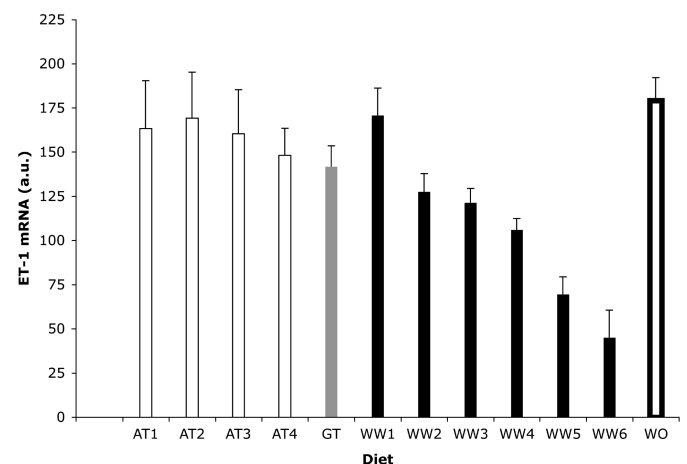

FIGURE 2 Aortic tissue ET-1 mRNA levels in hamsters fed high-fat diets containing various combinations of walnuts and tocopherols. Values are means ± SEM, *n* = 3 separate analyses of total mRNA isolated from a single pool per group. Statistical analyses were done using randomization and rank tests because of the limitations imposed by the sampling schema in the estimation of underlying uncertainties due to between-animal variation. Dietary α-tocopherol did not affect ET-1 mRNA, whereas increasing dietary walnuts had a significant, inverse, dose-dependent effect, *P* < 0.0028.

hamsters fed increasing concentrations of whole walnuts had a progressive decline ($P < 0.0028$) in ET-1 mRNA levels with the highest dose of walnuts (WW6) producing ET-1 mRNA levels 75% lower than those of AT1 or AT3, the lowest or normal α-tocopherol diet, respectively.

DISCUSSION

Ros and et al. (2) and Zhao et al. (3) both used walnuts as well as other fat sources to study the effects of walnut consumption, and both suggested that walnuts may have positive, nonplasma lipid (i.e., triacylglycerol and cholesterol)–related effects on endothelial function. The current study using atherosclerotic hyperlipidemic Golden Syrian hamsters targeted aortic ET-1 mRNA levels along with other atherosclerosis-related markers (SOD and aortic esterified cholesterol levels) to quantify the effects of walnuts on diet-induced atherosclerosis.

There was a dramatic decline in aortic tissue ET-1 mRNA abundance with increasing whole-walnut consumption, which is markedly different from the absence of any effect seen in diets formulated using different tocopherols (pure α- or γ-). These differences make it unlikely that the walnut effects on ET-1 mRNA levels are due to their high γ-T content. The higher mRNA levels in the lowest tocopherol diet group are consistent with the elevated aortic ET-1 protein expression associated with atherosclerosis reported by Matawari et al. (19). The pathways from ET-1 mRNA to ET-1 are complex (29), but multiple reports documented that ET-1 mRNA levels are coupled to ET-1 protein levels and that changes in mRNA levels are reflected by changes in ET-1 protein levels(11,30).

No alterations were seen in either aortic Mn SOD, Cu/Zn SOD, or biliverdin reductase protein levels, all proteins upregulated by oxidative stress (31–34). This suggests that the ET-1 effects were not due to changes in oxidative stress; it is noteworthy that both endothelial cell ET-1 protein and message are reported to be upregulated by increases in oxidative stress (11). The (n-3) fatty acids from walnuts are also unlikely to have affected ET-1 levels in the hyperlipidemic hamsters given both our efforts to balance fatty acids and previous reports indicating that they did not affect plasma cholesterol levels, oxidative stress, and the atherosclerotic process in hyperlipidemic rabbits nor do (n-3) fatty acids reduce human plasma ET-1 (35,36). The greater extent of the declines in ET-1 mRNA in the walnut groups relative to those seen in the tocopherol-added groups with comparable levels of tocopherols suggests that components other than tocopherols are involved. Although walnuts are a rich source of a variety of phytochemicals (37), the absence of any difference in terms of effect on aortic CE concentration between the walnut oil group and the comparable whole-walnut group (WO vs. WW1) suggests that the active elements for CE are located in the fat compartment of the walnut, which parallels the results for almond components whose effects on plasma lipid levels and LDL oxidizability appeared to be localized in the oil fraction (38).

Integrating the results of the human clinical walnut feeding studies (2,3) and the current study, the reduction in aortic ET-1 after walnut feeding provides further support for the suggestion that walnuts have effects on the endothelium. The walnut-related effects on ET-1, which were not accompanied by appreciable changes in the documented oxidative stress markers, may also provide an explanation for what appear to be paradoxical results from the human studies. Although walnut consumption improved endothelium-dependent vasodilation and reduced levels of vascular cell adhesion molecule-1 (VCAM-1), walnut feeding alone did not affect endothelium-independent vasodilation, levels of intercellular adhesion molecule-1 (ICAM-1), homocysteine, oxidation biomarkers, and, importantly, C-reactive protein (CRP) (2,3). The absence of any changes in either CRP or oxidative stress despite positive changes in CVD risk seems discordant given that both oxidative stress and CRP are strongly associated with atherothrombotic disease (39). A possible explanation for heart disease risk reduction after walnut consumption despite the absence of declines in either CRP or oxidative stress arises from the nature of ET-1–related processes. Increased ICAM-1 and VCAM-1 expression, monocyte chemoattractant chemokine-1 production, and a marked, sustained increase in native LDL uptake by macrophages upon incubation with CRP are mediated via ET-1 and IL-6 signaling. Moreover, these effects can be attenuated by blocking either endothelin receptors or inhibiting IL-6 activity with antibodies (40). More recently, Li et al. (41) demonstrated that simultaneous inhibition of ET-1 and IL-6 signaling attenuated the effect of CRP on the endothelial lectin-like oxidized LDL receptor-1 (LOX-1) in cultured human aortic endothelial cells, suggesting that LOX-1 induction by CRP requires both ET-1 and IL-6. This ET-1 connection to LOX-1 is noteworthy because endothelial LOX-1 is a major oxidized LDL receptor through which oxidized LDL induces endothelial dysfunction (42,43). Integrating the findings of Ros et al. (2), Zhao et al. (3), and the current study suggests that walnut consumption likely inhibits those CVD processes that result in endothelial dysfunction driven by ET-1 (44), whereas IL-6–driven processes such as ICAM-1 induction are minimally affected (45). Of note, Sabate et al. (46) reported very recently that increasing walnut consumption does not decrease CRP, but increased walnut consumption in vitamin E supplement users and in those with higher α-tocopherol:cholesterol ratios in fact raised CRP levels.

The oxidative hypothesis suggests that antioxidants in foodstuffs and/or specific components will decrease CVD risk by lowering oxidative stress; thus, increasing attention has been paid to antioxidants as a target. However, it is growing increasingly evident that antioxidants delivered either as supplements or in the context of a specific foodstuff or a diet differ markedly in their CVD-related effects. The well-documented beneficial effects of Mediterranean-style diets on endothelial dysfunction and markers of vascular inflammation (47) are in contrast to the absence of documented beneficial effects for individual components (48,49). In addition, 2 recent studies reported that tocopherol used as a supplement either provided no benefit or actually increased overall mortality risk or coronary heart failure, respectively (50,51). Finally, the health-related effects of the different tocopherol isomers are also a topic for ongoing research (52); the current study results showed that γ-T and α-T (GT compared with AT6) did not differ in terms of their effects on aortic CE concentration.

In summary, this study details large declines in aortic ET-1 with increasing walnut intake, suggesting that walnut consumption has beneficial effects on CVD risk in part via the ET-1–related effects on endothelial processes. These findings relating to ET-1 also provide a potential explanation for the benefits of walnut consumption for CVD risk despite the absence of any or only modest changes in several other CVD-related risk factors reported in clinical studies based on walnut consumption (2,3) via the involvement of ET-1 in mediating the effects of those other risk factors. Finally, the inability of tocopherol-related effects to account for the effects observed with walnuts when coupled with the disappointing findings in trials of tocopherol as an antioxidant supplement reemphasizes the need for increased understanding of how certain whole foods and diets are associated with heart health.

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